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NO DRAWINGS

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COMPLETE SPECIFICATION

New vaccines for combating influenza and the preparation thereof

We, RHONE-POULENC S.A., a French Body corporate, of 22 Avenue Montaigne, Paris 8e, France, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

The present invention relates to a process for the preparation of inactivated viral suspensions and of monovalent or polyvalent vaccines for combating influenza, and the new vaccines thus obtained.

The viruses used for preparing these vaccines are the human influenza viruses (Types A, A_1, A_2 or B), equine, porcine or fowl influenza viruses.

The process for the preparation of inactivated viral suspensions, in accordance with the present invention, consists of inoculating one or more strains of one or other of the viral agents mentioned above in the allantoic cavity of embryonated chicken eggs, cultivating the virus by incubation of the inoculated eggs, separating the viral suspension from the eggs and purifying it by centrifugation, and inactivating the virus in the purified suspension by treatment with diethyl ether or ethyl acetate at a temperature of from 0° to 5°C., thus maintaining the neuraminidase activity of the virus. The suspensions obtained, emulsified with an oily adjuvant of predetermined composition, give stable oily emulsions of inactivated influenza viruses which can be administered to human beings or to animals for protecting them against influenzal infections caused by the viruses characteristic of each of these species.

In practice, the particles of influenza viruses of the various antigenic types mentioned above are cultivated in accordance with the usual techniques in the allantoic cavity of the embryonated chicken egg and, after incubation for about 2 to 3 days, the allantoic liquids are drawn off and purified by differential centrifugation and filtration. In this way, there are obtained viral suspensions suitable for the inactivation treatment.

Inactivation of this viral suspension, with or without the addition of a buffering agent such as an isotonic phosphate solution, is effected by treatment with diethyl ether by the technique particularly described by F. M. Davenport and collaborators, J. Lab. Clin. Med. *63,5* (1964), so as to obtain haemagglutinating, antigenic but non-infectious subunits of the viral particles. The treatment with diethyl ether is effected by adding the ether to the previously obtained purified viral suspension (preferably employing two volumes of ether per unit volume of viral suspension) and stirring the mixture at a temperature of from 0° to 5°C. Afterwards, the suspension of haemagglutinating subunits is separated by decantation. Under these conditions, the neuraminidase activity of the viruses is preserved. The viral suspension is then checked by the usual procedures to ensure it is free from infectious influenza viruses and pyrogens. In the inactivation step, ethyl acetate can, with advantage, be used instead of diethyl ether.

For human or veterinary use, by subcutaneous or intramuscular route, the aqueous suspension of inactivated viral particles obtained by the process hereinbefore described is emulsified in a mixture of one or more vegetable or mineral oils, or hydrophilic natural triglycerides, and an appropriate emulsifying agent so as to obtain stable suspensions which can be easily administered. As the vegetable oil, it is preferred to use soya oil, but sesame, peanut and olive oil are also suitable. As the hydrophilic natural triglyceride, there may, for example, be employed polyoxyethylenated oleic triglycerides or polyoxyethylenated palmito-

stearic triglycerides. Mannitol oleate is preferably used as the emulsifying agent.

The emulsion is obtained by the usual methods, for example, by stirring rapidly the mixture of the inactivated viral suspension and two oily adjuvants at a temperature in the region of 0°C. In this way, an emulsion of haemagglutinating sub-units is obtained which can be used for immunising human beings or animals. The emulsion constitutes a vaccine which can be administered subcutaneously or intramuscularly.

The inactivated viral suspension can also be used directly; more particularly, it can be administered by the nasal route after atomisation by any appropriate means. The suspension can be stored in containers, such as atomiser bottles, with an appropriate gaseous or liquid propellent, enabling withdrawal from the container of a dispersion in the form of fine droplets or aerosols.

For use by the rectal route, the aqueous suspension of inactivated viral particles is lyophilised and the lyophilisate is incorporated into a fatty excipient which is usual for suppositories.

The process previously described is applicable to the preparation of monovalent and polyvalent vaccines against influenza. However, when polyvalent vaccines are desired, they are preferably prepared by mixing suspensions of separately obtained monotype haemagglutinating sub-units and then incorporating them into the oily adjuvant, as previously described.

The process of preparation as described above enables new vaccines against influenza to be obtained, which vaccines constitute an improvement in this field because of their ease of production with high yields and avoidance of dialyses, excellent immunising activity, the duration of the immunity produced, their ease of administration (subcutaneously, intramuscularly, nasally or rectally) and their absence of pyrogenic effect.

The following Examples illustrate the invention:

EXAMPLE I

The strain A_2/England/1/66 of A_2 influenza virus, or the strain B/Rumania/2/66 of B influenza virus, or the strain Ann Arbor/1/57 of A_1 influenza virus, or any other strain of human influenza virus of antigenic types A, A_1, A_2 or B obtained from the collection of the World Influenza Centre in London, is cultivated in the allantoic cavity of embryonated chicken eggs having 10 days of incubation. The inoculated eggs are incubated at 37°C. for 48 to 72 hours and then refrigerated at 4°C. for 18 hours, care being taken to remove beforehand the eggs of which the embryos are dead. After refrigeration, the allantoic liquids of the inoculated eggs are withdrawn aseptically by suction by means of a vacuum pump and combined in order to form a pool of 500 to 1000 cc. The quantity of virus present in this mixture is determined by the haemagglutination reaction and by titration *in ovo*. By varying, according to the influenza strain used, the dilution of the inoculum and the period of incubation of the inoculated eggs, it is attempted to obtain pools containing between 512 and 2048 haemagglutinating units per cc. (HAU/cc) of virus.

These pools are then clarified by centrifugation at low speed (about 1,000 G) for 15 minutes the supernatant substances are centrifuged for 60 minutes at 40,000 G in a preparative ultracentrifuge. The pellet obtained is brought into suspension homogeneously in a volume of isotonic phosphate buffering agent [pH 7.2; see R. Dulbecco and M. Vogt, J. Exp. Med. *99*, 167 (1954)].

This concentrated suspension is then purified by centrifugation in density gradients of potassium tartrate or sucrose [see C. E. Schwerdt and F. L. Schaffer, Virology *2*, 665 (1956); J. F. McCrea *et al*, Nature *189*, 220 (1961)], using a preparative ultracentrifuge. The gradient zones containing the highest content of HAU/cc are combined so as to obtain a viral suspension containing at least 5,000 HAU per mg of protein. This suspension can then be diluted in an isotonic phosphate buffering agent so as to contain from 1,000 to 2,000 HAU/cc of virus.

For treatment with diethyl ether in order to inactivate the virus, one volume of the purified viral suspension as obtained above has added to it two volumes of diethyl ether free from peroxide, and 1 mg. per cc. of polyoxyethylenesorbitan monooleate (Tween 80—"Tween" is a registered Trade Mark). This mixture is placed in a melting ice bath and continuously stirred by means of a blade-type agitator for 5 hours, the operation taking place under aseptic conditions. The ether and viral suspension, now composed of haemagglutinating sub-units, are separated by decantation and the residual ether is evaporated *in vacuo*. The amount of HAU/cc is determined by the usual method after this treatment; it is generally found that it is slightly higher than the original amount. The suspension of haemagglutinating sub-units is then filtered through cellulose ester membranes (mean dimension of pores: 0.45 microns) in order to eliminate any contaminating material.

The tests for bacterial and fungal sterility are carried out by the usual techiques on thioglycolate broth, nutrient agar medium of the National Institute of Health, Sabourand's agar nutrient medium and special enrichment broth for Mycoplasma. The absence of residual infectious influenza virus after treatment with diethyl ether is determined by two successive passages at different dilutions into the allantoic cavity of the embry-

onated chicken egg. The absence of pyrogenic effect is established by the usual technique by intravenous injection into a rabbit.

The bacteriologically sterile suspension, free from infectious influenza virus and pyrogens, is kept at 4°C. in sealed ampoules, after addition of sodium merthiolate ("Merthiolate" is a registered Trade Mark) to give a final concentration of 1 in 10,000.

The formation of an emulsion of the previously obtained suspension of haemagglutinating sub-units of influenza viruses in an oily adjuvant is carried out in the following manner:

To 30 cc. of the suspension, there are added 27 cc. of soya oil, 3 cc. of mannitol oleate and 0.5 cc. of polyoxyethylenesorbitan monooleate (Tween 80) which have all been previously sterilised. (The vegetable oils are sterilised by filtration through cellulose ester membranes: the mannitol oleate and the polyoxyethylenesorbitan monooleate are sterilised in an autoclave). While keeping the resulting mixture in a bath of melting ice, the rod of a turbine disperser is introduced aseptically into it, and is operated for 5 minutes. A white, creamy, homogeneous and stable emulsion is obtained, which is kept at 4°C. in flasks with rubber stoppers and equipped with metal caps.

The monovalent vaccine thus obtained is suitable for intramuscular or subcutaneous administration to man in a volume of 1 cc. (intramuscularly) or 0.4 cc. (subcutaneously).

EXAMPLE II

By operating as described in Example I and starting with the same strain of influenza virus, but using ethyl acetate as the inactivation agent, there is obtained a suspension of haemagglutinating sub-units of the initial virus, of which the controls are effected as described in Example I.

EXAMPLE III

For the preparation of polyvalent vaccines intended for administration to man by the intramuscular or subcutaneous route, the various strains of influenza virus of types A, A_1, A_2 and B which it is desired to incorporate into the vaccine are cultivated *in ovo*, purified by centrifugation and treated with diethyl ether or ethyl acetate, as described in Examples I and II. The various monotypic suspensions thus obtained are then mixed so that the final suspension contains 100 to 500 HAU/cc of each antigenic type represented. The controls for sterility, absence of residual infectious virus and absence of pyrogens, and emulsification in the oily adjuvant and the storage of the vaccine, are carried out as in Example I.

EXAMPLE IV

The preparation of anti-influenza vaccine intended for immunisation of the porcine species is effected as follows.

The strain A/Swine S_{15} of porcine influenza virus is cultivated *in ovo* and the virus is purified as described in Examples I and II, but limiting the purification to the first stage (centrifugation at 40,000 G for 60 minutes). The treatment with diethyl ether is effected as described in Example I. The final suspension should contain 500 to 1,000 HAU/cc of virus. The controls for sterility and absence of residual infectious virus are carried out as in Example I. Emulsification in the oily adjuvant is also effected as in Example I, but to the mixture, before its dispersion, there is added 5% of an interesterified natural triglyceride [such as Labrafil M 1944 C or Labrafil M 2735 (Gattefosse)]. The vaccine so obtained is suitable for administration by the intramuscular route, using a volume of 5 cc.

EXAMPLE V

The preparation of anti-influenza vaccine intended for immunisation of the horse species is carried out in the same way as in Example IV but using, as virus strains, the equine influenza viruses A/Equi/Prague and A/Equi/Miami, or strains which are antigenically related to them. The vaccine is bivalent, comprising a strain of Type A/Equi/Prague and a strain of Type A/Equi/Miami. As in the case of the porcine vaccine, the oily adjuvant (vegetable oil plus mannitol oleate) contains 5% of an interesterified natural triglyceride. The vaccine is suitable for intramuscular administration, using a volume of 5 cc.

EXAMPLE VI

The preparation of anti-influenza vaccine intended for immunising turkeys is carried out as in Examples IV and V but using, as the virus strain, the fowl influenza virus turkey/England/63 (Langham). The oily adjuvant, as in the case of the porcine and equine vaccines, contains 5% of an interesterified natural triglyceride. The vaccine is suitable for administration by the intramuscular route (using a volume of 1 cc.) or subcutaneously into the wing membrane (using a volume of 0.2 cc.).

EXAMPLE VII

The preparation of anti-influenza vaccine intended for administration to human beings by the intranasal route is carried out as in Examples I and II as regards the culture of the viruses *in ovo*, their concentration, their purification, their treatment with diethyl ether or ethyl acetate and the mixing of human strains representative of the types A, A_1, A_2 and B, so as to obtain a monovalent or polyvalent vaccine. The tests for sterility and absence of residual infectious virus are the same as in Examples I and II. After treatment with diethyl ether or ethyl acetate, the addition of sodium merthiolate to the suspensions of haemagglutinating subunits is omitted. It is assured that the subunits have maintained a neuraminidase en-

zymatic activity by making an aliquot part react with a preparation of mucoproteins extracted from the sub-maxillary gland of an ox and determining the liberation of N-acetylneuraminic acid from this substrate.

The monotype or polytype aqueous suspension of haemagglutinating sub-units is not emulsified in an oily adjuvant, as in the preceding Examples. It is placed in an atomising apparatus under nitrogen pressure and administered by atomisation into the nostrils.

EXAMPLE VIII

Suppositories intended for immunising human beings by the rectal route are prepared in the following manner.

The viruses of Type A, A_1, A_2 and B are cultivated *in ovo*, concentrated, purified and disintegrated with diethyl ether or ethyl acetate, as described in Examples I, II and III. The monotype or polytype aqueous suspensions of haemagglutinating sub-units obtained, to which sodium merthiolate is not added, are lyophilised and the dry product is incorporated into an interesterified hydrogenated palm oil preparation melted beforehand at 38°C., which is then left to solidify in appropriate moulds in order to form suppositories. The proportion of lyophilised suspension of haemagglutinating sub-units added to the mass for suppositories as described above is calculated in such a way that each suppository contains at least 1,000 haemagglutinating units of each virus strain represented in the vaccine.

In this specification reference is made to conditions maintaining the neuraminidase activity of the virus in the viral suspensions obtained by the process of the invention. The neuraminidase activity of a viral suspension of haemagglutinating sub-units is determined by reacting a suspension with a preparation of mucoproteins extracted from the sub-maxillary gland of the ox ("sialic acid concentrate", manufactured by Nutritional Biochemicals Corporation, Cleveland, United States of America) previously reduced by means of potassium borohydride. The enzymatic reaction of the suspension of haemagglutinating sub-units with this reduced mucin is effected on the water bath at 37°C. for 2 hours and the liberated N-acetylneuraminic acid is titrated by the thiobarbituric acid method described by Warren, J. Biol. Chem. *234*, 1971 (1959). The unit of neuraminidase activity is defined by the quantity of enzyme which, under these conditions, liberates in one minute 1 micromole of N-acetylneuraminic acid. In the case of the virus A_1/England/1/66, a suspension of haemagglutinating sub-units contains from 1 to 2 units of neuraminidase activity per litre, and in the case of the virus B/Rumania/2/66 it contains from 4 to 6 units per litre.

WHAT WE CLAIM IS:

1. Process for the preparation of monovalent of polyvalent inactivated viral suspensions of human, equine, porcine or fowl influenza viruses, which consists of cultivating in the allantoic cavity of embryonated chicken egg one or more strains of one or other of said viruses, separating the allantoic liquid containing the virus, purifying the viral suspension so obtained by centrifugation, and treating the thus purified suspension of the virus with diethyl ether or ethyl acetate at a temperature of from 0° to 5°C. to inactivate the virus.

2. Process according to claim 1 in which two volumes of diethyl ether or ethyl acetate are used per unit volume of viral suspension.

3. Process according to claim 1 or 2 in which the virus in suspension in an isotonic phosphate buffering agent is treated with diethyl ether or ethyl acetate.

4. Process according to claim 1, 2 or 3 in which the resultant inactivated viral suspension comprising haemagglutinating sub-units is separated by decantation.

5. Process for the preparation of polyvalent inactivated viral suspensions of human, equine, porcine or fowl influenza viruses, which comprises mixing two or more monovalent inactivated viral suspensions obtained by the process claimed in any one of claims 1 to 4.

6. Process according to any one of claims 1 to 5 followed by the step of lyophilising the obtained inactivated viral suspension.

7. Process for the preparation of monovalent or polyvalent inactivated viral suspensions according to claim 1 substantially as described in any one of Examples I to VIII.

8. Monovalent or polyvalent inactivated suspensions of human, equine, porcine or fowl influenza viruses when prepared by the process claimed in any one of the preceding claims.

9. Vaccines which comprise a monovalent or polyvalent inactivated suspension or composition of human, equine, porcine or fowl influenza viruses when prepared by the process claimed in any one of claims 1 to 7.

10. Vaccines according to claim 9 prepared by emulsifying the monovalent or polyvalent inactivated viral suspension with one or more vegetable or mineral oils, or hydrophilic natural triglycerides, and an emulsifying agent.

11. Vaccines according to claim 10 in which the viral suspension is emulsified in soya oil.

12. Vaccines according to claim 10 in which the viral suspension is emulsified in a polyoxyethylenated oleic triglyceride or polyoxyethylenated palmitostearic tri-

glyceride.

13. Vaccines according to claim 10, 11 or 12 in which mannitol oleate is employed as the emulsifying agent.

14. Vaccines according to claim 9 in a container permitting the withdrawal therefrom of a quantity of inactivated viral suspension in the form of fine droplets or aerosols.

15. Vaccines according to claim 9 prepared by lyophilising a viral suspension obtained by the process claimed in any one of claims 2 to 5, incorporation of the lyophilised product into a fatty excipient, and formation of suppositories.

16. Process for the preparation of vaccines substantially as hereinbefore described with especial reference to any one of Examples I and III to VIII.

J. A. KEMP & CO.,
Chartered Patent Agents,
14, South Square,
Gray's Inn,
London, W.C.1.

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